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Award Number: DAMD17-00-1-0710

TITLE: Behavior and Characterization of Prostatic Stem Cells

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REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20020816 053

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 00 - 30 Sep 01)	
4. TITLE AND SUBTITLE Behavior and Characterization of Prostatic Stem Cells			5. FUNDING NUMBERS DAMD17-00-1-0710	
6. AUTHOR(S) E. Lynette Wilson, Ph.D. Sarah Salm, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) New York University School of Medicine New York, New York 10016 E-Mail: wilsoe01@endeavor.med.nyu.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) The aim of this proposal is to identify prostate stem cells using a novel in vivo transplant approach and basal and luminal cell lines that we have established in culture. We also plan to transform the basal and luminal cell lines and study their behavior in vitro and in vivo to determine if transformed basal cells can give rise to luminal tumors in vivo as most prostatic tumors have a luminal phenotype. We have successfully tagged our basal and luminal cell lines with green fluorescent protein (GFP) and have established a novel in vivo intraprostatic transplant model using these tagged cell lines. We show that both basal and luminal cell lines incorporate into normal prostatic epithelium and that GFP-expressing basal cells give rise to GFP-expressing luminal cells in mouse prostatic ducts. These results show that both basal and luminal epithelial cells can home and engraft in specific niches when inoculated intraprostatically and that both cell types are capable of self-renewal indicating that they have stem cell features. They also show that basal cells can differentiate into luminal cells when inoculated intraprostatically. The identification of prostatic stem cells and their features is highly relevant as they are the targets of transformation resulting in prostatic carcinogenesis.				
14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) Prostate epithelial stem cell engraftment, Epithelial stem cell transformation and tumorigenicity				15. NUMBER OF PAGES 8
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

The aim of this proposal is to identify prostatic stem cells by a novel *in vivo* approach using prostatic basal and luminal cell lines that we have established in culture. We also plan to transform the basal and luminal cell lines and study the behavior of these *in vitro* and *in vivo* to determine whether transformed basal cells can give rise to luminal tumors *in vivo*, as most prostatic tumors have a luminal phenotype. The stem cells of any organ renew the tissue throughout the lifespan of the individual. They are long-lived cells that divide infrequently. Most of the cell division in an organ takes place in the post-stem cell compartment, the transit-amplifying (TA) cell population (Lavker and Sun, 2000; Slack, 2000). The identification and characterization of stem cells is important since they represent a major target of carcinogenesis as well as a potential source of benign prostatic hyperplasia (BPH) (De Marzo et al., 1998). A better understanding of the location and properties of prostatic stem cells may therefore help explain some puzzling features of the gland. For example, in human prostate, which can be divided anatomically into the central, transition and peripheral zones, carcinoma arises mainly in the peripheral zone whereas BPH develops largely in the transition zone (McNeal et al., 1988; De Marzo et al., 1998).

The location of prostatic stem cells is currently unknown. They are generally considered to lie within the basal compartment (Bonkhoff and Remberger, 1996; Hayward et al., 1999) although some evidence indicates that luminal cells may also have stem cell properties (Evans and Chandler, 1987b; Evans and Chandler, 1987a). An important feature of stem cells is their ability to engraft in their appropriate niche. The first aim of this proposal was to transfect our murine cell lines with GFP and to determine whether the basal and luminal cells can engraft intraprostatically in a novel prostate transplant assay that we proposed developing. Evidence of engraftment *in vivo* would indicate that the cell lines had stem cell properties.

Body

Task 1. Transfection of prostatic cell lines with a GFP-expression vector, pTracer-SV40.

We have established two prostatic epithelial cell lines. The basal cell line, PE-B-1, grows as an adherent monolayer with many round cells adhering loosely to the monolayer in confluent cultures (Fig 1 A). The cells express basal cytokeratin 14. (Fig 1B, C). The luminal cell line, PE-L-1, has a typical epithelial morphology (Fig 1D) and expresses luminal cytokeratin 8 (Fig 1E, F).

The basal and luminal cell lines have been stably transfected with the expression tracer vector pTracer-SV40 (Invitrogen, CA) which contains a green fluorescent protein (GFP) gene for detection of transfected cells. This vector was chosen since it also contains the gene for the selection antibiotic Zeocin rather than Neomycin. Our cell lines are Neomycin resistant, having been obtained from p53 null mice in which the p53 gene is disrupted by a Neomycin cassette. Basal and luminal cells were seeded at 1×10^3 cells/dish in 35 mm dishes and cultured overnight. Zeocin was then added to the cultures at 100, 250, 500, 750 and 1000 $\mu\text{g/ml}$. The cells were then cultured further, with medium and antibiotic replacement every 2 days. After 7 days cloning rings were placed around surviving antibiotic-resistant colonies, which were then removed from the parent dishes using trypsin digestion, after which they were re-seeded into 24 well plates. Clones were cultured further in the presence of antibiotic and those clones expressing GFP strongly as detected by fluorescent microscopy were selected for further use. Transfection efficiency was found to be low, with only approximately 1-2% of cells being successfully transfected. The cells also proved somewhat resistant to Zeocin and only the highest concentration of the antibiotic yielded isolated colonies. Stably transfected clones were obtained after 3 weeks in culture.

Clones that expressed significant levels of GFP were pooled to give a single population of GFP-expressing basal or luminal epithelial cells.

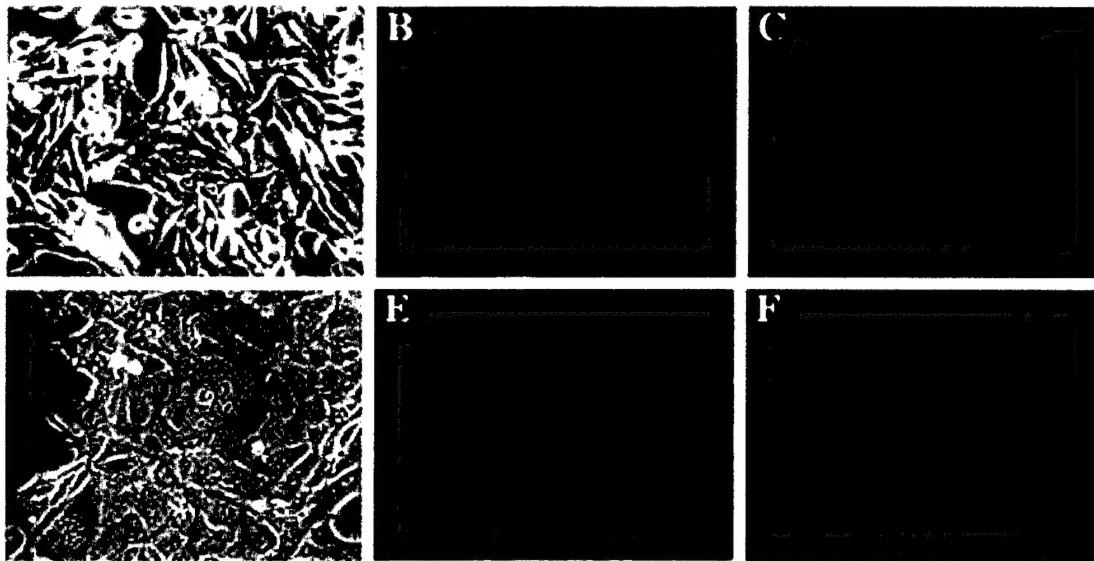


Figure 1. Phenotype of prostatic epithelial cell lines, PE-B-1 and PE-L-1.
A and D - Phase contrast micrographs of basal cell line PE-B-1 (A) and luminal cell line PE-L-1 (D).
B and E - Immunofluorescence micrographs showing expression of basal cytokeratin 14 by PE-B-1 (B) and luminal cytokeratin 8 by PE-L-1 (E), using FITC-labeled anti-CK antibodies.
C and F - Control wells stained with irrelevant antibodies.

We had originally planned to transfect GFP into an additional cell line that expressed both basal and luminal cytokeratins (line 12'). However, we had problems with the stability of this line in that even when cloned from single cells, the resulting cells did not all express both basal and luminal cytokeratins. After numerous attempts to obtain a uniform population of cells with stable expression of both cytokeratins were unsuccessful we proceeded with tasks 2 and 3 using the GFP-expressing basal and luminal cell lines.

Tasks 2 and 3. Inoculation and examination of GFP-expressing cells in vivo

Intraprostatic inoculation

Prostatic stem cells are generally considered to reside in the basal layer (Bonkhoff and Remberger, 1996; Hayward et al., 1999), although evidence of luminal cells being able to self-renew also exists (Evans and Chandler, 1987b; Evans and Chandler, 1987a). One of the characteristics of stem cells is their ability to home, to engraft and to proliferate in their micro-environmental "niches". The unequivocal identification of a stem cell requires a transplant model in which tagged candidate cells introduced into a recipient animal give rise to the appropriate differentiated progeny.

For this aim we developed a unique prostate transplant model using our GFP-expressing prostate cells. This is the first report of this type of prostate model. We injected our GFP-expressing cells into the prostates of adult animals. As stem cells require 'empty' niches into which they can engraft we reasoned that we would have more success if we transplanted cells into a regenerating prostate rather than an intact gland. We therefor

castrated adult mice and 8 days later we 'primed' the prostate by injecting testosterone propionate (4 μ g/g body wt) intraperitoneally. In addition, we implanted slow-release dihydrotestosterone (DHT) pellets subcutaneously. Twenty four hours later GFP-expressing basal and luminal cells were injected into each lobe of the dorsolateral prostate (DLP) (2.5×10^5 cells/lobe/ 10μ l). As we expected engraftment of very few cells, we induced several cycles of prostatic involution and regrowth in some animals by removing and replacing androgens in order to try and expand the numbers of GFP-expressing cells, thereby enhancing our chances of identifying them in random sections of prostatic tissue. Mice were therefore killed 10 days after engraftment and after 1, 2 and 3 cycles of androgen withdrawal and replacement and prostatic tissue examined for evidence of GFP-expressing cells using antibodies to GFP (Invitrogen, CA) and secondary anti-rabbit horse radish peroxidase (HRP)-labeled antibodies (Amersham, NJ) and the substrate NovaRED.

After 10 days evidence of engraftment was obtained for both the basal and luminal cell lines as GFP-expressing cells could be seen within the normal prostatic epithelium. GFP-expressing cells incorporated within prostatic epithelium persisted even after 3 successive cycles of androgen withdrawal and replenishment. This indicated that we were observing cells that had been inserted and incorporated into the epithelium of ducts, rather than cells that had merely survived the inoculation and that had remained within the lumen of ducts. Fig 2 represents a section through a prostatic duct of an animal that received GFP-labeled basal cells and it shows that GFP-expressing cells have been incorporated into a prostatic duct. Fig 2 also shows clear evidence that luminal cells also express GFP. As this animal was inoculated with a cloned basal GFP-expressing cell line that contained no luminal cells this indicates that prostatic basal cells can give rise to luminal cells in an in vivo intraprostatic transplant model.

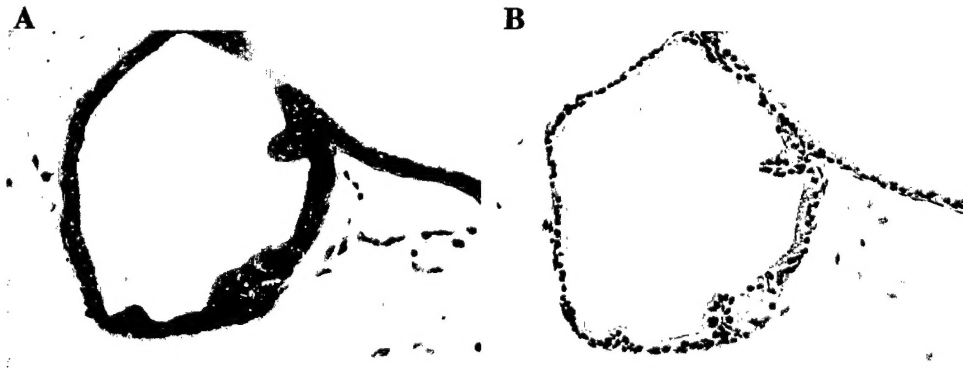


Figure 2. GFP-expressing basal cells were inoculated into involuted recipient prostates. Engrafted cells were detected using an anti-GFP antibody.
A - GFP-expressing cells were incorporated into the prostatic ducts and gave rise to GFP-expressing luminal cells.
B - control, in which an irrelevant primary antibody was used, confirming the specificity of the GFP antibody.

A basal cell line has been shown to differentiate into luminal cells when inoculated with urogenital sinus mesenchyme under the renal capsule (Hayward et al., 1999). Our experiments are the first to establish an in vivo prostate transplant model and to show basal cells give rise to luminal cells within a normal prostate indicating that basal cells can give rise to luminal cells in vivo.

Both basal and luminal cells were capable of engrafting into prostate tissue. Preliminary results show evidence of engraftment in 10/15 mice inoculated with basal cells and 18/19 mice inoculated with luminal cells. As we have only examined random sections of

prostatic tissue for GFP-expressing cells it is possible that the negative animals contained cells that had engrafted but that were not evident in the sections that we examined. A problem with our model is that it is not quantitative since we cannot measure the efficiency of engraftment. In the first instance each inoculation is 'random' as the cells are not inoculated into a particular niche but will be distributed throughout the prostatic tissue. The number of cells of the original inoculum that engrafted cannot therefore be ascertained. In addition, random sections of tissue may not always contain detectable engrafted cells.

Furthermore, it is very difficult to ascertain whether the inoculation of GFP-tagged luminal cells give rise to GFP-tagged basal cells by histological examination of tissue. The basal layer is very small in comparison with the luminal layer and basal cytoplasm is not readily evident in histological tissues. In order to address some of these concerns we are currently proceeding with experiments in which GFP-tagged cells are inoculated intraprostatically and in place of histological examination the prostate is removed, digested with collagenase and trypsin and the isolated cells examined for evidence of basal and luminal GFP expression. Cells will be examined concurrently for cytokeratin and GFP expression. This will conclusively show whether luminal cells can give rise to basal cells after in vivo engraftment. These experiments will also determine the numbers of GFP-expressing cells within the prostate as even if this number is very small, GFP cells will be detected immunocytochemically whereas they may not be detected in random sections of the gland.

Key research accomplishments

- 1) Basal and luminal prostatic cell lines transfected with GFP
- 2) In vivo prostatic transplant model established
- 3) Successful intraprostatic engraftment of GFP-tagged basal and luminal prostatic epithelial cells
- 4) Basal cells give rise to luminal cells intraprostatically

Reportable outcomes

We have developed GFP-tagged basal and luminal murine prostatic cell lines capable of in vivo engraftment in normal prostatic tissue. These cell lines are available for distribution.

Sarah Salm applied successfully for a DOD postdoctoral fellowship based partially on some preliminary data obtained from this project.

Conclusions

These results are relevant to prostate carcinogenesis for the following reasons. The identification of the stem cell compartment and the location of prostate stem cells is essential for the elucidation of the mechanisms that are involved in prostate carcinogenesis as stem cells are the targets of transformation. They are considered to reside in the basal layer so it is puzzling that prostate carcinomas generally have a luminal phenotype. Our experiments show that both basal and luminal cell compartments can independently self-renew and give rise to prostatic tissue in an in vivo prostate stem cell transplant assay. They also show that basal cells give rise to luminal cells. We do not currently know whether the engrafted luminal cells can give rise to basal cells but we are currently planning experiments to address this issue. The knowledge obtained from these studies will indicate whether basal and luminal compartments can independently self-renew and serve as targets for transformation. This knowledge will help design rational therapies for retarding or preventing the processes that result in the transformation of the stem cell compartment of the prostate.

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Personnel supported by grant

Sarah Salm Post doctoral fellow Effort 100%
E Lynette Wilson PI Effort 35%